1   Full title: *Borrelia afzelii* does not suppress the development of anti-tick immunity in

2   bank voles

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4   Running head: *Borrelia* and anti-tick immunity in vole

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20

21 **Abstract**

22 Vector-borne pathogens manipulate their vertebrate hosts to enhance their transmission to

23 arthropod vectors. The ability of vertebrate hosts to develop acquired immunity against

24 arthropod vectors represents an existential threat for both the vector and the pathogen.

25 The purpose of the study was to test whether the tick-borne spirochete bacterium *Borrelia*

26 *afzelii* could suppress the development of acquired immunity to its tick vector *Ixodes*

27 *ricinus* in the bank vole *Myodes glareolus*, which is an important host for both the tick

28 and the pathogen. We created a group of *B. afzelii*-infected bank voles and an uninfected

29 control group by exposing lab-reared animals to infected or uninfected ticks. At 1, 2, and

30 3 months post-infection, all bank voles were infested with larval *I. ricinus* ticks. The bank

31 voles developed a strong antibody response against tick salivary gland extract proteins.

32 This anti-tick immunity had negative effects on tick fitness traits including engorged

33 larval weight, unfed nymphal weight, larva-to-nymph molting time and larva-to-nymph

34 molting success. Infection with *B. afzelii* did not suppress the development of acquired

35 immunity against *I. ricinus* ticks. The development of anti-tick immunity was strongly

36 correlated with a dramatic temporal decline in both the bacterial abundance in the host

37 ear tissues and the host-tick transmission success of *B. afzelii*. Our study suggests that the

38 development of anti-tick immunity in bank voles has important consequences for the

39 density of infected ticks and the risk of Lyme borreliosis.

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41

42 **Importance**

43 Many pathogens enhance their persistence and transmission by suppressing the immune

44 system of their host. We used an experimental infection approach to test whether the

45 Lyme disease pathogen, *Borrelia afzelii*, could suppress the development of acquired

46 immunity against its tick vector (*Ixodes ricinus*) in the bank vole (*Myodes glareolus*), but

47 found no evidence for this phenomenon. Uninfected and *B. afzelii*-infected bank voles

48 both developed a strong IgG antibody response against tick salivary gland extract

49 following repeated infestations with *I. ricinus* ticks. The development of anti-tick

50 immunity was negatively correlated with the abundance of *B. afzelii* in ear tissue biopsies

51 and with host-to-tick transmission to *I. ricinus* ticks. Our study suggests that anti-tick

52 immunity in the bank vole reduces the prevalence of this important tick-borne pathogen.

53

54 **Key words**

55 Anti-tick immunity, bank vole, *Borrelia afzelii*, host-to-tick transmission,

56 immunosuppression, *Ixodes ricinus*, Lyme disease, *Myodes glareolus*, tick-borne disease,

57 tick salivary gland extract

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Introduction

60

61   Many pathogens exploit the obligate blood-feeding habits of hematophagous

62   arthropods to achieve transmission between vertebrate hosts. These vector-borne

63   pathogens and their arthropod vectors have co-evolved an intimate relationship that is

64   characterized by specificity and adaptive complexity (1-3). Specificity is shown by the

65   fact that many vector-borne pathogens are only effectively acquired and transmitted by

66   one or a few closely related vector species (4-6). Examples of adaptive complexity in

67   pathogens include increasing the vector's production of immunosuppressive saliva

68   molecules to enhance their own transmission (7), protecting the vector against changes in

69   the environment (8, 9), and manipulating the odor profile of the vertebrate host to

70   increase host-vector encounter rates and pathogen transmission (10-12). Thus, whenever

71   it suits their interests, we expect vector-borne pathogens to enhance the fitness of their

72   arthropod vectors.

73   From the perspective of the pathogen and vector, the ability of the vertebrate host

74   to develop immunity or resistance against the vector represents a shared existential threat.

75   Compared to other hematophagous arthropods, this threat of inducing acquired resistance

76   is particularly important for ticks, probably because they feed on the host in large

77   numbers and for longer periods of time (several days). Studies on a variety of host-tick

78   systems have shown that this anti-tick immunity or acquired tick resistance can reduce

79   tick fitness components including measures of larval engorgement, duration of

80   attachment, percentage of recovered larvae, and larva-to-nymph molting success (13-16).

81   Acquired immunity against ticks also enhances the host defense against tick-borne

82   pathogens (17). Acquired immunity against ticks can impair transmission of tick-borne

83 pathogens, establishment of the pathogen within the host, and alter the cutaneous

84 environment at the tick attachment site (17-19). In summary, the development of acquired

85 immunity in the vertebrate host against ticks reduces the fitness of both the tick vector

86 and the tick-borne pathogen.

87   Ticks and specifically tick salivary glands and tick saliva have evolved to cope

88 with this existential threat of host resistance. Tick saliva contains nearly 500 proteins and

89 peptides that belong to at least 25 different protein families (20). These tick saliva

90 molecules have powerful anti-haemostatic, anti-inflammatory and immunomodulatory

91 properties (21) that seek to subvert the host immune system at every turn. This powerful

92 pharmacopeia also enhances the transmission of pathogens, a phenomenon known as

93 saliva-assisted transmission (22). Conversely, a number of tick-borne pathogens have

94 developed a variety of strategies that allow them to evade or suppress the host immune

95 system (23). In turn, pathogen-induced immunosuppression may reduce the development

96 of anti-tick immunity and thereby enhance tick feeding success.

97   To study whether vector-borne pathogens can suppress the development of

98 acquired immunity against the arthropod vector in the vertebrate host, we used a tick-

99 borne spirochete bacterium belonging to the *B. burgdorferi* sensu lato (sl) genospecies

100 complex and that causes Lyme borreliosis (LB) in humans as a model system (24, 25).

101 Our study was motivated by a recent demonstration that *B. burgdorferi* sensu stricto (ss)

102 could suppress the development of acquired immunity in the lab mouse *Mus musculus*

103 (26). We wanted to test whether other *B. burgdorferi* sl genospecies could induce

104 immunosuppression in their natural rodent reservoir hosts. The most common LB system

105 in Europe includes the bacterium *B. afzelii*, the tick *Ixodes ricinus*, and rodent hosts such

106   as the bank vole (*Myodes glareolus*) (27-30). The bank vole is a good host for *B. afzelii*

107   (27-29), but it develops acquired immunity against *Ixodes* ticks (14, 27). Previous

108   workers have suggested that this anti-tick immunity in bank voles reduces the production

109   of *B. afzelii*-infected ticks (27).

110   The purpose of this study was to test whether *B. afzelii* could inhibit the

111   development of acquired anti-tick immunity in bank voles. We created *B. afzelii*-infected

112   bank voles and uninfected bank voles, and repeatedly infested them with larval *I. ricinus*

113   ticks to induce acquired anti-tick immunity. We predicted that bank voles would develop

114   a strong antibody response against tick salivary gland proteins, which would decrease

115   tick fitness. We expected that infection with *B. afzelii* would suppress the development of

116   acquired anti-tick immunity in bank voles. We therefore expected tick fitness to be higher

117   on the *B. afzelii*-infected bank voles compared to the uninfected controls.

118

119   **Materials and Methods**

120   **Bank voles, *Ixodes ricinus* ticks and *Borrelia afzelii*:** The bank voles came from

121   a laboratory colony at the University of Neuchâtel. This colony was descended from bank

122   voles that had been captured at a field site near Neuchâtel, Switzerland in the summer of

123   2014 (31). All animals used in the study were lab-born, 5 to 10 weeks old at the start of

124   the study, and not infected with *B. afzelii*. During the experiment, bank voles were

125   maintained in individual cages and were given food and water *ad libitum*. The *I. ricinus*

126   ticks came from the University of Neuchâtel colony, and larval *I. ricinus* ticks were also

127   purchased from Insect Services (Germany). Bank voles were experimentally infected via

128   tick-bite with *B. afzelii* isolate NE4049. This isolate was originally obtained from an *I.*

6

129   *ricinus* tick in Neuchâtel, it has multi-locus sequence type 679, *ospC* major group (oMG)

130   A10, and strain ID number 1887 in the *Borrelia* MLST database. We used isolate

131   NE4049 because our previous work has shown that it is highly infectious to *I. ricinus*

132   ticks and rodents including bank voles (31-34). *B. afzelii* strains carrying oMG A10 also

133   have the highest frequency in wild *I. ricinus* populations near Neuchâtel (35).

134   **Ethics statement and animal experimentation permits:** The study was

135   performed at the University of Neuchâtel and followed the Swiss legislation on animal

136   experimentation. The commission that is part of the 'Service de la Consommation et des

137   Affaires Vétérinaires (SCAV)' of canton Vaud evaluated and approved the ethics of this

138   part of the study. The SCAV of canton Neuchâtel issued the animal experimentation

139   permits for the study (NE1/2017) and for the maintenance of the *I. ricinus* tick colony at

140   the University of Neuchâtel (NE5/2014).

141   **Creation of nymphs infected with *B. afzelii*:** Larval *I. ricinus* ticks were fed on

142   BALB/c mice (*Mus musculus*) that had been previously infected with *B. afzelii* isolate

143   NE4049 via tick-bite. The resultant engorged larval ticks were collected, stored in

144   individual Eppendorf tubes, and allowed to molt into nymphs. A random sample of 30

145   nymphs was tested for *B. afzelii* using qPCR, and the prevalence of infection was 93.3%

146   (28 infected/ 30 total). To create uninfected control nymphs, larval *I. ricinus* ticks were

147   fed on uninfected BALB/c mice and the resultant engorged larvae were allowed to molt

148   into nymphs.

149   **Experimental design:** Forty voles were randomly assigned to one of two

150   experimental groups: uninfected control (n = 20) and infected with *B. afzelii* (n = 20).

151   Each vole in the uninfected control group was infested with 5 uninfected nymphs,

7

152 whereas each vole in the *B. afzelii*-infected group was infested with 5 *B. afzelii*-infected

153 nymphs. During the nymphal challenge, voles were anesthetized with a

154 ketamine/xylazine cocktail via intraperitoneal injection (1:2:9 xylazine: ketamine: PBS;

155 dose of 5 μl per 10 g of vole body mass). Six of the 40 voles died under anesthesia

156 leaving 34 animals. To prevent the voles from removing the nymphs via grooming, the

157 nymphs were placed in a plastic capsule attached to a shaved area on the back of the vole.

158 Each vole was fitted with a collar to prevent the animal from removing the capsule. The

159 capsules were checked on a daily basis and the engorged nymphs were collected and

160 tested for their *B. afzelii* infection status. The infection status of the voles was confirmed

161 using additional diagnostic criteria (see below).

162 **Larval infestations:** Voles were infested with larval ticks on three separate

163 occasions at 27, 54, and 84 days post-infection (PI). For the first and second infestation,

164 we used larvae from our *I. ricinus* tick colony at the University of Neuchâtel. For the

165 third infestation, we had a shortage of larvae at our colony, and we therefore purchased

166 them from Insect Services (Germany). For each infestation, the voles were anaesthetized

167 and approximately 100–150 larvae were put on each vole. For the first infestation, voles

168 were anesthetized with the ketamine/xylazine cocktail described previously.

169 Unexpectedly, of the remaining 34 voles, another 6 died under anesthesia leaving 28

170 animals. For the second and third infestations, voles were anesthetized with 2%

171 isoflurane using the Combi-vet® anesthesia system (Rothacher Medical, Switzerland)

172 and no additional animals were lost. For each vole, an ear biopsy and a blood sample

173 (from the saphenous vein) were collected on four occasions at -1, 26, 51, and 106 days

174 PI. Ear tissue biopsies and serum samples were kept at –20°C for future analysis. On day

175   106 PI, the voles were euthanized with CO_2 and exsanguinated. Of the 28 voles that

176   survived to the end of the study, there were 14 in the uninfected control group and 14 in

177   the *B. afzelii*-infected group.

178   **Tick phenotypes:** Engorged larval ticks detached from voles after 2–4 days of

179   blood feeding. For each of the 84 combinations of infestation and vole, a maximum of 50

180   engorged larvae were collected and placed in individual 1.5 ml Eppendorf tubes. The

181   tubes contained a strip of moistened paper towel to maintain a high humidity. The

182   engorged larvae were kept in an incubator (Sanyo, Japan) with a day-night cycle of 16

183   hours of light and 8 hours of darkness, and a relative humidity of 85% (section 1 in the

184   supplementary material). For each infestation and each vole, a maximum of 20 engorged

185   larvae were randomly selected and weighed within 5 days of drop off (total of 1680

186   engorged larvae). Once the engorged larval ticks started molting, they were checked two

187   or three times per week until the percentage of molted nymphs reached 80%. The median

188   molting time was defined as the date when 50% of the engorged larvae had molted into

189   nymphs. At two weeks after the median molting time, the ticks that had been weighed as

190   larvae were weighed again as nymphs. At four weeks after the median molting time, 10

191   nymphs were randomly selected for each of the 84 combinations of vole and infestation

192   (total of 840 nymphs), and these nymphs were frozen at –80°C to assess their *B. afzelii*

193   infection status.

194   Different tick phenotypes were used to determine the effect of acquired anti-tick

195   immunity and *B. afzelii* infection on tick fitness. The tick phenotypes included: engorged

196   larval weight, unfed nymphal weight, molting time and molting success. Molting time

197   was defined as the number of days between the detachment of the engorged larvae and

198   the molting to unfed nymphs. Molting success was the percentage of engorged larvae that

199   molted into flat nymphs.

200   **ELISA and qPCR:** The serum samples of the voles were tested for the presence

201   of *B. afzelii*-specific IgG antibodies using a commercial ELISA assay as previously

202   described (31). The DNA was extracted from the vole tissue samples, the engorged

203   nymphs, and the flat nymphs as previously described (31, 36). All the DNA extractions

204   were eluted into 65 μl of water. The vole ear tissue samples and the ticks were tested for

205   infection with *B. afzelii* using a qPCR assay that targets a 132 bp fragment of the *flagellin*

206   gene as previously described (31). For each vole tissue sample and tick, 3.0 μl of DNA

207   template was used in the qPCR reaction.

208   **Culture of viable *B. afzelii* spirochetes from xenodiagnostic nymphs:** To

209   demonstrate that the voles transmitted a viable *B. afzelii* infection to the *I. ricinus* ticks,

210   flat nymphs (that had fed as larvae on the voles) were cultured in BSK-H media

211   (Bio&Sell). Six nymphs were randomly selected for each bank vole (total of 168

212   nymphs). Each tick was washed with 70% ethanol, cut in half, and placed in an individual

213   1.5 ml Eppendorf tube containing BSK-H medium. The culture tubes were kept at 34°C

214   in an incubator and were screened for motile *B. afzelii* spirochetes over a period of 4

215   weeks using a dark field microscope.

216   **Tick salivary gland extract protein solution:** Tick salivary gland extract (SGE)

217   proteins were obtained from the salivary glands of engorged adult female *I. ricinus* ticks

218   from our laboratory colony. A total of 28 female and 5 male adult ticks were fed on one

219   rabbit. After 96 hours of blood feeding, the 24 engorged female ticks were removed from

220   the rabbit with tweezers, washed with 70% ethanol, and washed with 1x PBS. The tick

221   salivary glands were dissected on ice using a dissecting microscope, washed with 1x

222   PBS, pooled and homogenized in 400 µl of 1x PBS, and kept at –80 °C. The tick salivary

223   gland cells were lysed by keeping the suspension on ice and conducting three short pulse

224   sonication sessions (5 s pulse, 30 s pause) using a SKAN sonifier 450 (SKAN AG,

225   Switzerland). Soluble antigens were obtained after centrifugation at 20,000 g for 15 min.

226   The protein concentration was determined using the Bradford assay. The concentration of

227   the tick SGE sample was 1.62 mg/ml for a total of 648 µg of tick SGE.

228   **Tick salivary gland extract ELISA:** The IgG antibody response of the voles

229   against tick SGE was measured using a homemade ELISA. 96-well tissue culture plates

230   (Fisher Scientific, Switzerland) were coated overnight at 4°C with 1 µg of tick SGE

231   protein per well. Wells were washed 3 times with PBS-Tween 0.1% between each change

232   of solution. The wells were incubated with a BSA 2% blocking solution for 2 hours,

233   followed by the bank vole serum samples (diluted 1:100 in 1x PBS) for 45 minutes, and

234   the secondary antibody for 45 minutes (diluted 1:5000 in 1x PBS). The secondary

235   antibody was a goat anti-*Mus musculus* IgG conjugated to horseradish peroxidase

236   (Promega). After adding 100 µl of TMB product (Promega) to each well, the absorbance

237   was measured at 652 nm every 2 minutes for one hour using a plate reader (Synergy HT,

238   Multi-detection plate reader, Bio-Tek, United States). The strength of the IgG antibody

239   response against tick SGE was determined for each serum sample by calculating the area

240   under the curve of the optical density (OD) versus time.

241   **Infection status of bank voles:** A vole was considered to be infected with *B.*

242   *afzelii*, if it tested positive for one or more of four criteria: (1) optical density > 500

243   absorbance units indicating the presence of *B. afzelii*-specific IgG antibodies, (2)

11

244   spirochete load in the ear tissue biopsy > 0, (3) spirochete load in the xenodiagnostic

245   nymphs > 0, and (4) culture of live spirochetes from xenodiagnostic nymphs. Thirteen of

246   the 14 voles in the *B. afzelii*-infected group tested positive for 3 or 4 criteria, and these 13

247   voles were therefore considered as infected with *B. afzelii* (section 2 in the supplementary

248   material). The vole that tested negative for all five criteria was excluded from the

249   analysis. As expected, all of the 14 voles in the control group tested negative for the 4

250   infection criteria. All statistical analyses are therefore based on 13 *B. afzelii*-infected

251   voles and 14 uninfected voles.

252

253   **Statistical Analysis**

254   All statistical analyses were done in R version 1.0.143 (R Development Core

255   Team 2015-08-14). Means are reported with their 95% confidence intervals (95% CI).

256   Normal versus binomial response variables were modelled using linear mixed effects

257   models (LMMs) with normal errors versus generalized linear mixed effects models

258   (GLMMs) with binomial errors, respectively. To determine the statistical significance of

259   each fixed factor, pairs of models that differed in the fixed factor of interest were

260   compared using log-likelihood ratio (LLR) tests. To calculate the p-value, the change in

261   deviance between models is compared to the Chi-square distribution. Bank vole identity

262   was included as a random factor.

263

264   **Tick life history traits**

265   The response variables included four tick life history traits: (1) engorged larval

266   weight, (2) unfed nymphal weight, (3) molting duration, and (4) molting success. The

267   engorged larval weight and unfed nymphal weight were log10-transformed to improve

268   the normality of the residuals. All response variables followed a normal distribution,

269   except molting success, which followed a binomial distribution. Each response variable

270   was modelled as a function of two fixed factors: infection status (two levels: uninfected

271   control and infected with *B. afzelii*), infestation number (two levels: first and third

272   infestation), and their interaction. For infestation number, only the first and third

273   infestations were used in the analysis to simplify the presentation of the results (full

274   analyses in section 4 of the supplementary material). We also modelled the tick SGE-

275   specific IgG antibody levels in the voles (log10-transformed) using an LMM model. The

276   fixed factors were infection status, day of blood sample (four levels: -1, 26, 51, and 106

277   days PI), and their interaction. The background variation in the OD values was corrected

278   for each plate as follows: the mean OD of the BSA controls for a given plate was

279   subtracted from the OD values of the bank vole serum samples in that plate.

280

281   **Relationships between *B. afzelii* infection phenotypes in the infected voles**

282   For the subset of infected voles, we had measured three *B. afzelii* infection

283   phenotypes around the time of each larval infestation (27, 54, and 84 days PI): (1) tick

284   SGE-specific IgG antibody levels of the vole (hereafter the anti-tick IgG response), (2)

285   spirochete load in the vole ear biopsy (number of spirochetes in the entire ear tissue

286   biopsy), and (3) host-to-tick transmission (percentage of flat nymphs that were infected

287   with *B. afzelii*). The spirochete loads in the ear biopsies were log10-transformed to

288   improve their fit to a normal distribution. The anti-tick IgG response and the log10(ear

289   tissue spirochete load) followed a normal distribution, whereas host-to-tick transmission

13

290   followed a binomial distribution. We conducted two separate analyses that were based on

291   causal relationships between these three variables. First, we modelled the ear biopsy

292   spirochete load as a function of the anti-tick IgG response. Second, we modelled host-to-

293   tick transmission as a function of the anti-tick IgG response and the ear biopsy spirochete

294   load.

295

296   **Results**

297   **Collection of engorged nymphal ticks from the voles:** We collected 47

298   engorged nymphs from the 14 voles in the control group (mean = 3.36, range = 1–5

299   engorged nymphs per vole). As expected, none of these nymphs tested positive for *B.*

300   *afzelii*. We collected 46 engorged nymphs from the 14 voles in the *B. afzelii*-infected

301   group (mean = 3.29, range = 0–5 engorged nymphs per vole). Our qPCR assay found that

302   58.7% (27/46) of the engorged nymphs were infected with *B. afzelii*. For 12 of the 14

303   voles in the infected group, we collected at least 1 engorged *B. afzelii*-infected nymph

304   (mean = 1.93, range = 1–4; Table S1 in the supplementary material). These data show

305   that the voles in the infected group and the control group were infested with similar

306   numbers of nymphs.

307

308   **Life history traits of *I. ricinus* ticks that fed on bank voles**

309   **Weight of blood-engorged larvae:** The engorged larval weight decreased from

310   the first to the third infestation (Figure 1). The LMM analysis of engorged larval weight

311   found a significant interaction between infection status and infestation number (LLR: $\chi_2 =$

312   8.316, df = 2, p = 0.016). Infestation number had a significant effect on the engorged

313   larval weight (LLR: χ_2 = 45.996, df = 2, p < 0.001), but the effect of infection status was

314   not significant (LLR: χ_2 = 0.028, df = 1, p = 0.866). For the first, second, and third larval

315   infestation, the mean engorged larval weight was 483 μg (95% CI: 475–492), 452 μg

316   (95% CI: 444–461), and 456 μg (95% CI: 448–465), respectively. Compared to the first

317   infestation, the mean engorged larval weight in the second and third infestation was

318   reduced by 6.4% (p = 0.006) and 5.6% (p < 0.001), respectively.

319   **Weight of the unfed nymphs:** The flat nymphal weight decreased from the first

320   to the third infestation (Figure 2). The LMM analysis of flat nymphal weight found no

321   significant interaction between infection status and infestation number (LRR: χ_2 = 5.299,

322   df = 2, p = 0.071). After removing the interaction, infestation number had a significant

323   effect on the flat nymphal weight (LRR: χ_2 = 74.807, df = 2, p < 0.001), but the effect of

324   infection status was not significant (LRR: χ_2 = 0.656, df = 1, p = 0.418). For the first,

325   second, and third larval infestation, the mean nymphal weight was 188 μg (95% CI: 183–

326   192), 166 μg (95% CI: 162–171), and 177 μg (95% CI: 172–181), respectively.

327   Compared to the first infestation, the mean flat nymphal weight in the second and third

328   infestation was reduced by 11.7% (p < 0.001) and 5.9% (p < 0.001), respectively.

329   **Molting time of engorged larval ticks to nymphs:** The larva-to-nymph molting

330   time was defined as the number of days between the drop-off of the engorged larval ticks

331   and the molt into flat nymphs. The molting time was monitored for a total of 2611

332   engorged larval ticks, of which 83.80% (2188/2611) molted into nymphs. The molting

333   time decreased over the three successive larval infestations (Figure 3). The LMM

334   analysis of molting time found no significant interaction between infection status and

335   infestation number (LRR: χ_2 = 4.341, df = 2, p = 0.114). After removing the interaction,

15

336 infestation number had a significant effect on the molting time (LRR: $\chi_2 = 246.7$, df = 2,

337 p < 0.001), but the effect of infection status was not significant (LRR: $\chi_2 = 0.004$, df = 1,

338 p = 0.946). For the first, second, and third larval infestation, the mean molting time was

339 51 days (95% CI: 50–53), 47 days (95% CI: 45–49), and 35 days (95% CI: 33–37),

340 respectively. Compared to the first infestation, the mean molting time in the second and

341 third infestation was reduced by 7.8% (p < 0.001) and 31.4% (p < 0.001), respectively.

342   **Larva-to-nymph molting success:** The molting success was defined as the

343 percentage of engorged larval ticks that molted into flat nymphs. The molting success

344 decreased over the three successive larval infestations (Figure 4). The GLMM analysis of

345 molting success found no significant interaction between infection status and infestation

346 number (LRR: $\chi_2 = 2.276$, df = 2, p = 0.320). After removing the interaction, infestation

347 number had a significant effect on the molting success (LRR: $\chi_2 = 35.121$, df = 2, p <

348 0.001), but the effect of infection status was not significant (LRR: $\chi_2 = 1.489$, df = 1, p =

349 0.222). For the first, second, and third larval infestation, the mean molting success was

350 88% (95% CI: 86–90%), 87% (95% CI: 84–91%), and 78% (95% CI: 75–81%),

351 respectively. Compared to the first infestation, the mean molting success in the second

352 and third infestation was reduced by 1.1% (p = 0.883) and 11.4% (p < 0.001),

353 respectively.

354

355 **Bank voles developed a strong IgG antibody response against the salivary gland**

356 **extract of *I. ricinus* ticks**

357 The tick SGE-specific IgG antibody levels (hereafter the anti-tick IgG response)

358 increased over the duration of the study (from day -1 to day 106;

16

359   Figure 5) and this change was significant (LME: χ_2 = 166.61, df = 1, p < 0.001).

360   For day -1, day 26, day 51, and day 106, the mean anti-tick IgG response (measured in

361   absorbance units) was 1176 (95% CI: 1040–1328), 1559 (95% CI: 1370–1774), 2391

362   (95% CI: 2112–2706), and 6351 (95% CI: 5635–7157), respectively. Compared to day -

363   1, the mean anti-tick IgG response on day 106 had increased 5.4-fold.

364   The LMM analysis of the anti-tick IgG response found that the interaction between

365   infection status and the day of the blood sample was significant (LRR: χ_2 = 12.638, df =

366   3, p = 0.005). We therefore tested the effect of infection status on the anti-tick IgG

367   response separately for each day. This analysis found a significant difference between the

368   infected group and the control group at day -1 (t-test: df = 25, t = 6.818, p < 0.001), day

369   26 (t-test: df = 22, t = 2.218, p = 0.037), day 51 (t-test: df = 24, t = 3.1036, p = 0.004), but

370   not at day 106 (t-test: df = 25, t = 0.117, p = 0.908). Thus, the anti-tick IgG response

371   reached similar levels in both groups of bank voles at the end of the experiment. On day

372   26 and day 51, the mean anti-tick IgG response of the infected group was 17.4% and

373   45.6% higher than the control group, respectively (

374   Figure 5). On day -1, the mean anti-tick IgG response of the infected group was 55.6%

375   lower than the control group (

376   Figure 5).

377

378   **Abundance of *B. afzelii* in ear biopsies and host-to-tick transmission**

379   **Spirochete load in the bank vole ear biopsy:** The spirochete loads in the vole

380   ear biopsies (2 mm diameter) decreased over the three infestations (section 5 in the

381   supplementary material). For the first, second, and third larval infestation, the mean

382   spirochete load in the ear biopsy (measured in number of spirochetes) was 5883 (95% CI:

383   1516–22834), 202 (95% CI: 52–786), and 13 (95% CI: 3–50), respectively. The anti-tick

384   IgG response had a significant negative effect on the spirochete load in the ear biopsy

385   (Figure 6; LME: χ_2 = 17.748, df = 1, p < 0.001; slope ± S.E. = -2.26 ± 0.537).

386   **Host-to-tick transmission of *Borrelia afzelii*:** After combining the three

387   infestations, host-to-tick transmission was 38.5% (144 infected nymphs/ 374 total

388   nymphs). Host-to-tick transmission decreased over the three infestations (section 6 in the

389   supplementary material). For the first, second, and third larval infestations, the host-to-

390   tick transmission was 59.4% (76/128), 35.6% (42/118), and 20.3% (26/128), respectively.

391   For 30.8% (4/13) of the voles, the host-to-tick transmission was 0% by the third

392   infestation. The anti-tick IgG response had a significant negative effect (GLME: χ_2 =

393   5.407, df = 1, p = 0.020; logit slope ± S.E. = -1.49 ± 0.639), whereas the log10-

394   transformed spirochete load in the ear biopsy had a significant positive effect on host-to-

395   tick transmission (Figure 7; GLME: χ_2 = 10.369, df = 1, p = 0.001; logit slope ± S.E. =

396   0.52 ± 0.162).

397

398   **Discussion**

399   **Ability of *B. burgdorferi* sl to suppress acquired immunity in the vertebrate**

400   **host:** Infection with *B. afzelii* in a natural rodent host did not prevent the development of

401   acquired immunity against larval *I. ricinus* ticks and its negative effects on tick life

402   history traits. Our study contrasts with a recent study showing that *B. burgdorferi* ss

403   suppressed the development of acquired immunity in laboratory *Mus musculus* mice (26).

404   Specifically, this study found that infected mice immunized with the influenza vaccine

18

405   did not develop protective antibodies against the influenza virus (26). The mechanism of

406   immunosuppression is that *B. burgdorferi* ss migrates to the mouse lymph nodes where it

407   inhibits the development of long-lived plasma cells and memory B cells, (26).

408   Differences between our study and (26) include the genospecies of *B. burgdorferi* sl (*B.*

409   *burgdorferi* ss versus *B. afzelii*), the antigen (influenza vaccine versus live ticks), the

410   rodent host (lab mice versus natural host), and mode of infection (needle inoculation

411   versus tick bite). Other tick-borne pathogens such as *Anaplasma phagocytophilum* and

412   *Babesia microti* have been documented to induce immunosuppression in vertebrate hosts

413   (23, 37). Interestingly, the protozoan parasite *B. microti* was able to induce

414   immunosuppression in laboratory mice, but not in the bank vole or any known natural

415   reservoir host (37, 38). These studies suggest that the ability of *B. burgdorferi* sl to

416   suppress the adaptive immune system may depend on the identity of the vertebrate host.

417   From an ecological and public health perspective, pathogen-induced

418   immunosuppression is important because it can facilitate the infection and emergence of

419   opportunistic pathogens (39). For example, the ability of HIV to suppress the immune

420   system has led to the re-emergence of tuberculosis in human populations in the

421   developing world (40). Community ecology studies often find positive associations

422   between *B. burgdorferi* sl genospecies and other tick-borne pathogens (39). For example,

423   the emergence of *B. microti* was strongly associated with the prevalence of *B.*

424   *burgdorferi* ss in the northeast United States (41, 42). In addition, *B. burgdorferi* sl

425   genospecies and strains within a genospecies have been found to be positively associated

426   (43-45). In summary, *B. burgdorferi* sl-induced immunosuppression in the vertebrate host

427   can facilitate mixed infections and the emergence of other tick-borne pathogens.

428   **Development of acquired immunity against ticks in vertebrate hosts:** Our

429 study found that repeated infestations with larval *I. ricinus* ticks caused the bank voles to

430 develop a strong IgG antibody response against the SGE proteins of *I. ricinus*. The

431 development of this anti-tick immunity was correlated with a reduction in tick fitness

432 including the size of the engorged larval ticks and the resultant flat nymphs, the duration

433 of the larva-to-nymph molt, and the larvae-to-nymph molting success. One complication

434 with our study was that the *I. ricinus* larvae for the first and second infestation came from

435 the University of Neuchâtel colony and the larvae for the third infestation came from

436 Insect Services (Germany). This happened because the University of Neuchâtel colony

437 failed to produce sufficient numbers of larvae for the third infestation. As a result, any

438 differences in tick phenotype between the first and third infestation could be due to innate

439 differences between these two tick colonies rather than acquired anti-tick immunity.

440 However, the statistically significant decreases in engorged larval weight and flat

441 nymphal weight between the first and second larval infestation (which used larvae from

442 the University of Neuchâtel colony) suggests that our bank voles developed acquired

443 anti-tick immunity after the first larval infestation.

444   Our results are in agreement with previous studies showing that bank voles

445 develop anti-tick immunity to larval ticks of *I. ricinus* or *I. trianguliceps* (14, 27, 37).

446 These studies found that anti-tick immunity reduced the percentage of fully engorged

447 larvae, larval engorgement index, duration of attachment, percentage of recovered larvae,

448 and larva-to-nymph molting success (14, 27, 37). The mechanism by which anti-tick

449 immunity reduces tick fitness is that it impairs tick blood feeding and therefore reduces

450 the quality and quantity of the blood meal (21). With respect to body size, previous

20

451   studies on *I. ricinus* have shown that larger nymphs have higher fat reserves (46) and that

452   such nymphs can quest for longer periods of time, which increases their chances of

453   finding a host in the field (9, 47, 48). Body size also influences female fecundity, with

454   larger female ticks laying larger clutches of eggs (49). The reduced molting success is the

455   most direct fitness cost because for arthropods such as ticks, the inability to complete the

456   molt is equivalent to death (50). Population ecology models of *Ixodes* ticks have shown

457   that the tick population growth rate is highly sensitive to molting success (51, 52). In

458   summary, the negative effects of acquired anti-tick immunity on tick life history traits can

459   reduce the abundance of ticks in the field.

460   **_B. burgdorferi_ sl pathogens establish chronic infections in rodents with high**

461   **lifetime host-to-tick transmission:** We showed that *B. afzelii* isolate NE4049

462   established a long-lived systemic infection in the bank voles. At the time of sacrifice, all

463   of the 13 bank voles showed systemic infections where the dorsal skin, ventral skin, ear,

464   bladder and heart were all infected with *B. afzelii*. These results are in agreement with

465   previous studies that have shown that *B. burgdorferi* sl pathogens established chronic

466   systemic infections in their rodent reservoir hosts (27, 33, 53, 54). For vector-borne

467   pathogens such as *B. burgdorferi* sl, chronic infections are adaptive because they increase

468   the lifetime transmission success of the infection (24, 33, 55). In the present study, host-

469   to-tick transmission of *B. afzelii* decreased three-fold over time from 60% in the first

470   infestation (4 weeks PI) to 20% in the third infestation (12 weeks PI). Less dramatic

471   declines have been observed in another important rodent reservoir, the wood mouse

472   (*Apodemus sylvaticus*), where host-to-tick transmission of *B. afzelii* decreased from 100%

473   at 3 weeks PI to ~40% at 9 weeks PI (54). A study that used the same isolate of *B. afzelii*

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474   (NE4049) and the same colony of *I. ricinus* ticks to infect *M. musculus* mice found that

475   host-to-tick transmission decreased from 90.8% at 5 weeks PI to 68.9% at 13 weeks PI

476   (33). Likewise, experimental infection studies on *B. burgdorferi* ss have shown that the

477   pattern of host-to-tick transmission over time can vary depending on the particular

478   combination of strain and rodent species (56-58). In general, host-to-tick transmission of

479   *B. burgdorferi* sl pathogens decreases over time in most rodent species, but the bank

480   voles in this study showed a particularly steep decline.

481   **Relationship between pathogen abundance in host tissues and host-to-vector**

482   **transmission:** For vector-borne pathogens, there is a direct relationship between the

483   pathogen's abundance in the relevant tissues and host-to-vector transmission. For

484   example, this relationship has been shown in rodent malaria where the parasite density in

485   the blood is critical for mouse-to-mosquito transmission (59). For *B. burgdorferi* sl

486   pathogens, the skin rather than the blood is the critical tissue for host-to-tick transmission

487   (60, 61). Field studies on *B. afzelii* in bank voles and other wild rodents found a positive

488   relationship between the spirochete load in the ear tissues and transmission to larval *I.*

489   *ricinus* ticks (62). Similarly, an infection experiment with laboratory *M. musculus* mice

490   and the *B. afzelii* strain used in the present study (NE4049), also found a positive

491   relationship between the spirochete load in the ears and the host-to-tick transmission (36).

492   Studies on laboratory *M. musculus* mice have shown that the spirochete load in the host

493   tissues can change over time (63-65), but these studies have not investigated host-to-tick

494   transmission. The present study found that the spirochete load of *B. afzelii* in the ear

495   biopsies decreased 450-fold over time from 5883 spirochetes per biopsy in the first

496   infestation (4 weeks PI) to 13 spirochetes per biopsy in the third infestation (12 weeks

497   PI). This decline in spirochete load was strongly correlated with the temporal increase of

498   anti-tick immunity. Our study suggests that an increasingly effective host immune

499   response (against the tick and/or the pathogen) reduced the spirochete load in the skin of

500   the bank voles, which subsequently reduced transmission of *B. afzelii* to feeding larval

501   ticks.

502   **Acquired immunity against *B. burgdorferi* sl and *Ixodes* ticks:** Rodents

503   develop strong antibody responses against *B. burgdorferi* sl pathogens, which are

504   believed to play an important role in controlling the infection (66-68). For example,

505   SCID mice that cannot produce antibodies have spirochete loads that are an order of

506   magnitude higher than immunocompetent mice, and inoculation of SCID mice with *B.*

507   *burgdorferi* sl anti-serum reduces the tissue spirochete load (64, 69-71). Previous studies

508   on bank voles and *Apodemus* mice suggested that the strength of the spirochete-reactive

509   antibody response was important for controlling host-to-tick transmission (29). The bank

510   voles in this study developed a strong IgG antibody response against *B. afzelii* as shown

511   by the results from the commercial ELISA. Thus, one plausible explanation is that the *B.*

512   *afzelii*-targeted antibody response was able to reduce the spirochete load in the skin and

513   thereby reduce host-to-tick transmission. A second explanation is that the anti-tick

514   immunity developed against the sequential larval infestations reduced host-to-tick

515   transmission success of *B. afzelii*. Previous studies have shown that the development of

516   acquired immunity against ticks in the vertebrate host has important consequences for the

517   acquisition and transmission of tick-borne pathogens (17, 18). We found a highly

518   significant negative relationship between the anti-tick IgG antibody response and host-to-

519   tick transmission, even after controlling for the temporal decline in spirochete load in the

23

520   ear tissues. This observation suggests that anti-tick immunity caused the temporal decline

521   in host-to-tick transmission. A plausible mechanism for this phenomenon is that the anti-

522   tick antibodies opsonize the tick salivary gland proteins and thereby transform the

523   feeding lesion into an immunologically hostile environment where spirochete survival

524   and spirochete migration to the mouthparts of feeding larval ticks are compromised (72-

525   74). A third explanation for the decline in host-to-tick transmission was that anti-tick

526   immunity shortened the feeding time of the larval ticks, which reduced the probability of

527   acquiring *B. afzelii*. A recent manipulative study showed that there was a positive

528   relationship between the duration of the blood meal of *I. scapularis* larvae and the

529   probability of host-to-tick transmission of *B. burgdorferi* ss (75). In summary, anti-

530   spirochete immunity, anti-tick immunity, and shorter feeding times of larval ticks are

531   three alternative explanations for the observed temporal decline in the ear tissue

532   spirochete loads and host-to-tick transmission of *B. afzelii*.

533   **Ecological consequences of acquired immunity in bank voles and associated**

534   **reductions in tick life history traits and host-to-tick transmission:** Several studies

535   have suggested that the bank vole is an important reservoir host of *B. afzelii* (14, 27, 29,

536   76-79). Early studies on the vertebrate host community in Sweden suggested that the

537   bank vole was the second-most important host of *B. afzelii* and produced 17% of the

538   infected *I. ricinus* nymphs (76). In this experimental study, we found that the anti-tick

539   immunity developed by bank voles had negative consequences for both the tick vector

540   and the tick-borne pathogen. Population ecology models of *I. ricinus* have shown that the

541   tick population size and growth rate are highly sensitive to density-dependent mortality

542   on the host, which can be mediated by acquired immunity (52). A recent field study in

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543   France found that the abundance of bank voles had negative effects on the abundance of

544   *I. ricinus* nymphs the following year (80). The explanation was that acquired immunity in

545   bank voles had a negative effect on the recruitment of feeding larval ticks into nymphs

546   the following year. Theoretical models have shown that the reproduction number (R_0) of

547   *B. burgdorferi* sl pathogens is strongly influenced by the duration of the infection and the

548   host-to-tick transmission rate (81). Both of these life history traits were strongly reduced

549   in the present study showing the importance of acquired immunity in controlling the

550   ecology of *B. burgdorferi* sl pathogens and the epidemiology of Lyme borreliosis.

551   **Conclusions:** We found no evidence that infection with *B. afzelii* suppressed the

552   development of acquired immunity against *I. ricinus* ticks in bank voles. Future research

553   needs to confirm whether suppression of acquired immunity is a widespread strategy in

554   the *B. burgdorferi* sl genospecies complex or whether it is restricted to certain strains or

555   unnatural hosts, such as laboratory mice. Repeated infestations with larval *I. ricinus* ticks

556   induced the bank voles to develop a strong IgG antibody response against the salivary

557   gland extract proteins of *I. ricinus*. This anti-tick antibody response reduced tick fitness

558   and was also associated with a dramatic temporal decline in the ear spirochete load and

559   host-to-tick transmission. Our study suggests that acquired anti-tick immunity in the

560   vertebrate host can play an important role in controlling the abundance of *B. burgdorferi*

561   sl-infected nymphs and hence the risk of Lyme borreliosis.

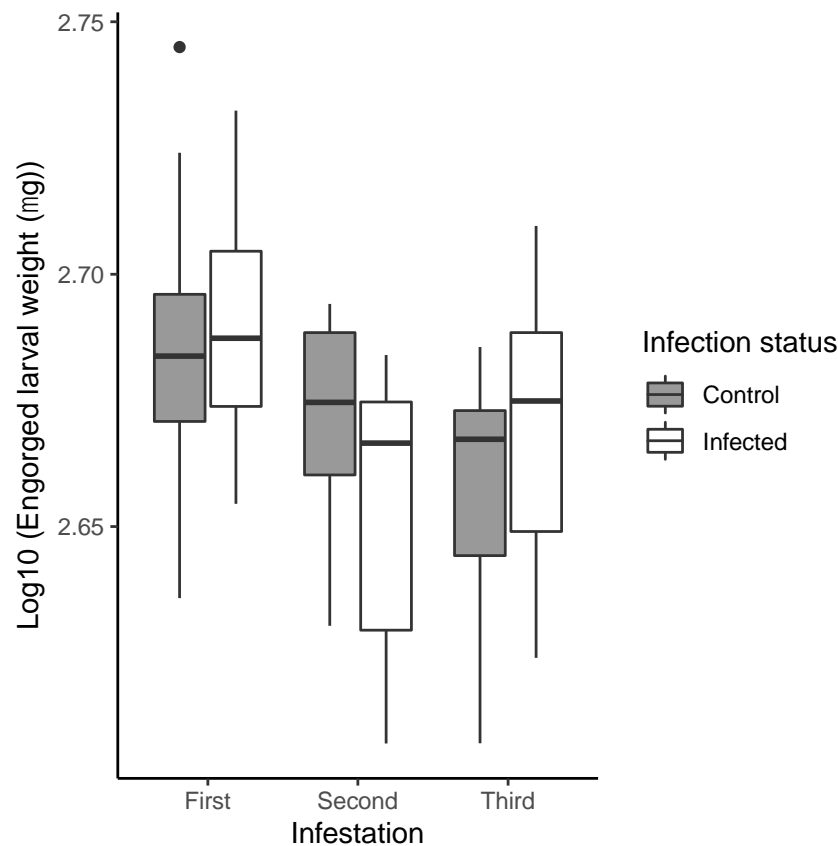562   FIGURES

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566   Figure 1. The engorged weight of *I. ricinus* larval ticks feeding on the bank voles

567   decreased over the three successive infestations. Infection of bank voles with *B. afzelii*

568   did not affect the engorged larval weight (measured in μg). The bank voles in the *B.*

569   *afzelii*-infected group (n = 13) and the control group (n = 14) were infested with larval

570   ticks at 27, 55, and 84 days PI. Each data point represents the mean engorged larval

571   weight for an individual bank vole and is based on ~20 ticks. Shown are the medians

572   (black line), the 25th and 75th percentiles (edges of the box), the minimum and maximum

573   values (whiskers), and the outliers (circles).

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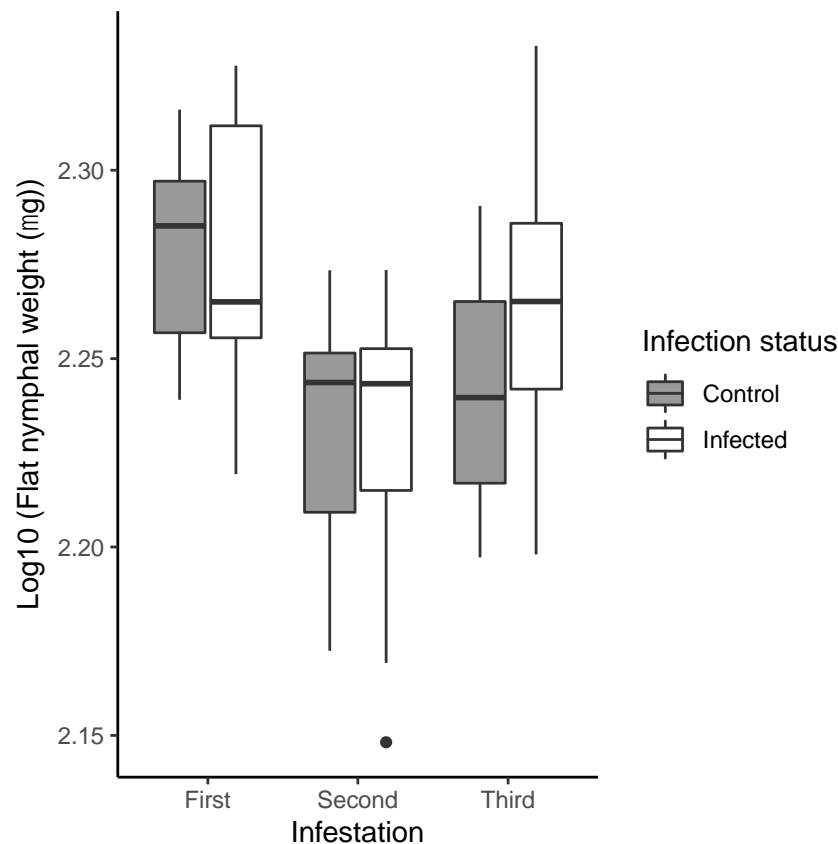
576 Figure 2. The weight of the flat *I. ricinus* nymphs decreased over the three successive

577 infestations. The flat nymphs had fed on the bank voles during the larval stage. Infection

578 of bank voles with *B. afzelii* did not affect the flat nymphal weight (measured in μg). The

579 bank voles in the *B. afzelii*-infected group (n = 13) and the control group (n = 14) were

580 infested with larval ticks at 27, 55, and 84 days PI. Each data point represents the mean

581 flat nymphal weight for an individual bank vole and is based on ~20 flat nymphs. Shown

582 are the medians (black line), the 25th and 75th percentiles (edges of the box), the

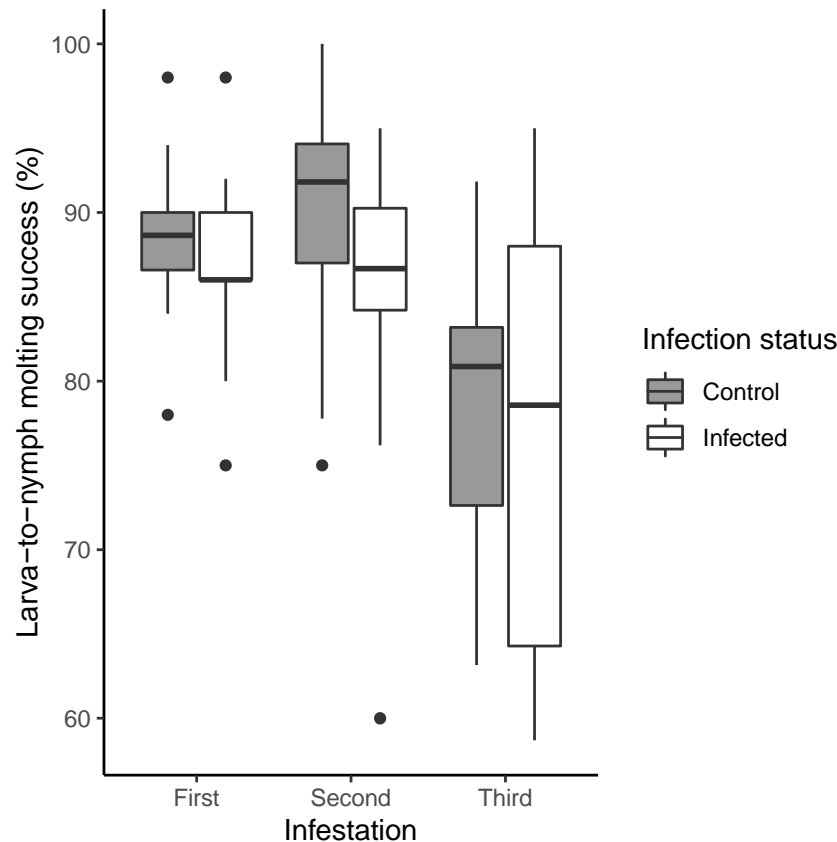583 minimum and maximum values (whiskers), and the outliers (circles).

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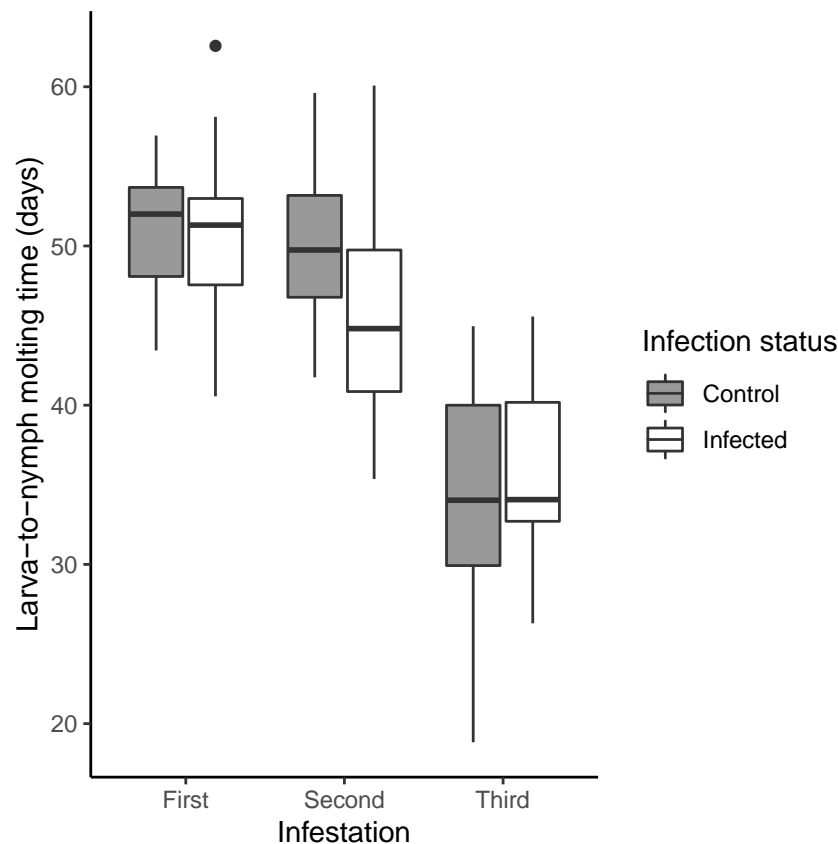
588   Figure 3. The larva-to-nymph molting time of *I. ricinus* ticks decreased over the three

589   successive infestations. The molting time refers to the number of days for an engorged

590   larval tick to molt into a nymphal tick. Infection of bank voles with *B. afzelii* did not

591   affect the molting time. The bank voles in the *B. afzelii*-infected group (n = 13) and the

592   control group (n = 14) were infested with larval ticks at 27, 55, and 84 days PI. Each data

593   point represents the mean larva-to-nymph molting time for an individual bank vole and is

594   based on ~50–100 ticks. Shown are the medians (black line), the 25th and 75th

595   percentiles (edges of the box), the minimum and maximum values (whiskers), and the

596   outliers (circles).

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600　Figure 4. The molting success of *I. ricinus* larval ticks decreased over the three

601　successive infestations. Molting success refers to the percentage of engorged larval ticks

602　that developed into the nymphal stage. Infection of bank voles with *B. afzelii* did not

603　affect the molting success. The bank voles in the *B. afzelii*-infected group (n = 13) and

604　the control group (n = 14) were infested with larval ticks at 27, 55, and 84 days PI. Each

605　data point represents the mean larva-to-nymph molting success for an individual bank

606　vole and is based on ~50–100 ticks. Shown are the medians (black line), the 25th and

607　75th percentiles (edges of the box), the minimum and maximum values (whiskers), and

608　the outliers (circles).

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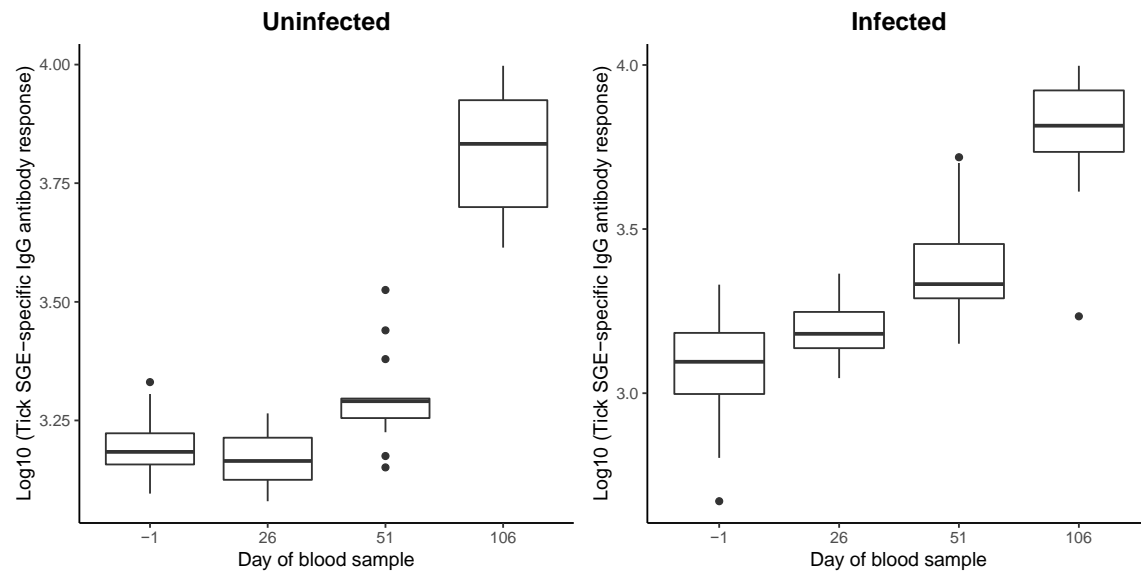
Figure 5. The bank voles developed a strong IgG antibody response against the salivary

gland extract of *I. ricinus* ticks over the three successive larval infestations in both the

control group and the *B. afzelii*-infected group. Serum samples were taken on -1, 26, 51,

and 106 days PI, which corresponded to time points before the nymphal challenge, and

after the first, second, and third larval infestation. The strength of the IgG antibody

response against tick salivary gland extract was measured as the absorbance from an

ELISA. Each data point represents the log10-transformed absorbance value for an

individual bank vole. Shown are the medians (black line), the 25th and 75th percentiles

(edges of the box), the minimum and maximum values (whiskers), and the outliers

(circles).

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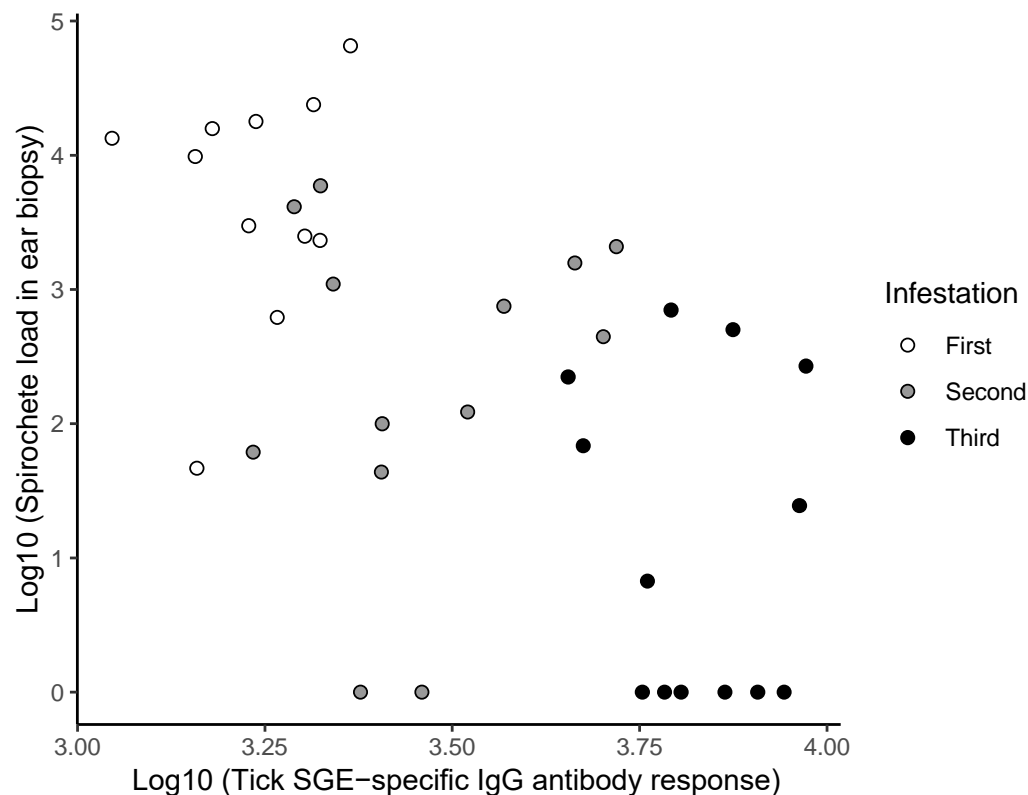
625   Figure 6. The spirochete load in the bank vole ear biopsy is negatively related to the tick

626   SGE-specific IgG antibody response. Data are shown for the subset of *B. afzelii*-infected

627   bank voles (n = 13 individuals) at the time of the first (open white circles), second (solid

628   grey circles), and third infestation (solid black circles). Ear tissue biopsies were taken at

629   26, 51, and 106 days PI and the serum samples were taken at 26, 51, and 106 days PI.

630   The 39 data points represent the 13 *B. afzelii*-infected bank voles at each of the three

631   infestations. Spirochete loads refer to the number of spirochetes in the whole ear tissue

632   biopsy (2 mm diameter).

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Figure 7. Host-to-tick transmission is positively correlated with spirochete load in the ear tissue of the bank voles. Host-to-tick transmission is the percentage of nymphs that acquired the *B. afzelii* infection during the larval blood meal. Data are shown for the subset of *B. afzelii*-infected bank voles (n = 13 individuals) at the time of the first (open white circles), second (solid grey circles), and third infestation (solid black circles). Ear tissue biopsies were taken at 26, 51, and 106 days PI and the bank voles were infested with larval ticks at 27, 55, and 84 days PI. The 39 data points represent the 13 *B. afzelii*-infected bank voles at each of the three infestations. Spirochete loads refer to the number of spirochetes in the whole ear tissue biopsy (2 mm diameter).

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652

Author contributions

654   A.G.-C. and M.J.V designed the study. A.G.-C., Y.L. and A.S performed the

655 experimental infections and the molecular work. O.R. created the larvae necessary for the

656 infestations. A.G.-C. analysed the data. A.G.-C. and M.J.V wrote the manuscript. All

657 authors read and approved the final version of the manuscript.

658

659 Competing interests: The authors declare no competing of interest.

660

Supplementary information

662   Additional information is contained in the supplementary information file.

663

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